

# The *Bcr-Abl* leukemia oncogene activates Jun kinase and requires Jun for transformation

(chronic myelogenous leukemia/mitogen-activated protein kinase)

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**ABSTRACT** The leukemogenic tyrosine kinase fusion protein *Bcr-Abl* activates a Ras-dependent pathway required for transformation. To examine subsequent signal transduction events we measured the effect of *Bcr-Abl* on two mitogen-activated protein kinase (MAPK) cascades—the extracellular signal-regulated kinase (ERK) pathway and the Jun N-terminal kinase (JNK) pathway. We find that *Bcr-Abl* primarily activates JNK in fibroblasts and hematopoietic cells. *Bcr-Abl* enhances JNK function as measured by transcription from Jun responsive promoters and requires Ras, MEK kinase (MAPK/ERK kinase), and JNK to do so. Dominant-negative mutants of c-Jun, which inhibit the endpoint of the JNK pathway, impair *Bcr-Abl* transforming activity. These findings implicate the JNK pathway in transformation by a human leukemia oncogene.

Receptor tyrosine kinases (RTKs) use a complex set of molecules conserved through evolution to transduce signals from outside the cell to the nucleus (1, 2). Early steps involve activation of Ras by exchange factors such as SOS or C3G in signaling complexes assembled by adaptor molecules such as Grb-2 and SHC (3). Subsequently, a serine/threonine kinase cascade involving mitogen-activated protein kinases (MAPKs) is activated. The best characterized of the MAPK pathways is the Ras/Raf/MEK/ERK pathway (ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase) (1). ERKs can activate transcription factors such as Elk-1/SAP-1 by phosphorylation at critical regulatory sites (2). Studies of the c-Jun transcription factor have uncovered a second MAPK pathway involving Jun N-terminal kinase (JNK) or stress-activated protein kinase (SAPK) (4–6). Analogous to the Raf/MEK/ERK pathway, JNK is activated by a dual specificity serine/threonine kinase (SEK1, MKK4, or JNKK), which, in turn, is activated by the MEKK (MEK kinase) serine/threonine kinase (7–11, 48). Two transcription factors, c-Jun (4, 5) and ATF-2 (12), are known substrates for JNK. Activation of the MEKK/SEK/JNK pathway leads to phosphorylation of c-Jun (13) or ATF-2 (12) on critical serine or threonine residues and enhances their activity as transcription factors. As with the ERKs, JNK is activated in a Ras-dependent fashion by RTKs (4, 11). These observations have led to a model for RTK signaling in which Ras may serve as an input signal for activation of both ERK and JNK pathways (10, 11). Factors that control the relative activation of each pathway are unknown and could influence the outcome of the RTK signal by affecting the range of transcription factors that become active.

Chronic myelogenous leukemia is a human leukemia associated with the t(9;22) Philadelphia chromosome translocation that creates the *Bcr-Abl* fusion gene (14). Depending on the breakpoint, *Bcr-Abl* encodes a 210- or 185-kDa cytoplasmic tyrosine kinase, which causes leukemia in murine model

systems (15, 16). c-Abl is also fused to retroviral Gag sequences in a murine leukemia caused by the *v-Abl* oncogene (17). Expression of *Bcr-Abl* leads to activation of Ras (18–20), and interruption of Ras function impairs *Bcr-Abl* transforming activity (20, 21). Similar to RTKs, *Bcr-Abl* appears to activate Ras through the assembly of signaling complexes containing adaptor molecules and GDP/GTP exchange factors. The precise details have not been elucidated, but *Bcr-Abl* can form complexes with Grb-2 (19, 22), SHC (23), and Crkl (24, 25). The events subsequent to Ras activation by *Bcr-Abl* are unclear. Complementation studies have shown that transformation by *Bcr-Abl* requires the activation of multiple pathways (26), one of which involves the transcription factor c-Myc.

Based on these similarities to RTKs, we reasoned that *Bcr-Abl* might also activate MAPK pathways. We therefore examined the relative activation of ERK and JNK by *Bcr-Abl*. We find that *Bcr-Abl* preferentially activates JNK. *Bcr-Abl* also enhances the activity of Jun-responsive promoters through a Ras- and JNK-dependent pathway, and dominant-negative mutants of Jun inhibit the transforming activity of *Bcr-Abl*. The results demonstrate preferential activation of the JNK pathway by a human leukemia tyrosine kinase and implicate this pathway in transformation.

## MATERIALS AND METHODS

**Cell Lines, Plasmids, RNA, and Protein Analysis.** Murine myeloid DAGM cells expressing *Bcr-Abl* were derived as described (27). AP-1-dependent transcription assays were performed by transient transfection of 293 cells using a reporter containing three copies of the tetradecanoyl phorbol acetate response element (TRE) present in the collagenase promoter fused to the chloramphenicol acetyltransferase gene (3× TRE-CAT) (28). Expression plasmids included *Bcr-Abl*, *v-Abl*, and Asn17 Ras in the pSRαMSVtkNeo vector (21), pSRαMEKKΔ K432M (11), pcDNA3 DNJNK1 (12), pEXV-3 Raf K375A (29), and pMV-Src (30). Cytoplasmic RNA was extracted as described (31) and analyzed by Northern blot using a murine Jun cDNA (32) as probe. *Bcr-Abl* or *v-Abl* protein expression was measured by Western blotting with anti-Abl pex-5 antibody.

**ERK and JNK Assays.** Transient assays for ERK and JNK activation were performed by calcium phosphate-mediated cotransfection of 293 cells using the plasmid pMX-139 encoding the full-length p42 human *ERK2* gene (provided by M. Raines, University of California, Los Angeles) and the plasmid pcDNA3 Flag-JNK1 (12), respectively. Cells were lysed in

Abbreviations: JNK, Jun N-terminal kinase; RTK, receptor tyrosine kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MEKK, MEK kinase; CAT, chloramphenicol acetyltransferase; MBP, myelin basic protein; GST, glutathione S-transferase; GM-CSF, granulocyte-macrophage colony-stimulating factor; TRE, tetradecanoyl phorbol acetate response element.

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buffer A (12) and incubated with anti-ERK antibody (a gift from C. Marshall, Institute of Cancer Research, London) (33) or anti-Flag antibody (Kodak) prebound to protein A-Sepharose beads. Immune complex kinase assays were performed as described (11, 12). Endogenous JNK assays were performed in similar fashion using anti-JNK antibody provided by R. Davis (University of Massachusetts).

**Soft Agar Transformation Assays.** cDNAs encoding mutant Jun and Fos proteins obtained from I. Verma (Salk Institute) (32) were subcloned into the retrovirus vector pSR $\alpha$ MSVtkNeo (34). Retrovirus stocks were prepared by transient transfection of 293 T cells with the ecotropic  $\psi$ -packaging plasmid as described (34). Indicator lines were generated by infection with the appropriate retrovirus stock and selection for 2–3 weeks in the antibiotic G418. Individual clones were pooled at the end of G418 selection and analyzed as populations. Transformation was measured by infecting the indicator lines with retrovirus stock expressing Bcr-Abl and plating the cells in soft agar within 48 hr in duplicate plates as described. Colonies >0.5 mm in diameter after 2 weeks were scored positive.

## RESULTS

**Bcr-Abl Preferentially Activates JNK.** We first compared the relative activation of ERK and JNK by Bcr-Abl in 293 human embryonic kidney cells to allow high-level expression of multiple plasmids in transient transfection experiments. ERK activation was measured by cotransfection of a vector expressing the human p42 ERK2 protein with a second plasmid expressing either the neomycin-resistance gene (*neo*) alone, v-Abl, p185 Bcr-Abl, or v-Src as a positive control (35). After 48 hr, ERK2 activity was measured by immune complex kinase assay using myelin basic protein (MBP) as a substrate. JNK activity was measured similarly except that a vector expressing an epitope-tagged JNK1 protein (FLAG-JNK1) was substituted for ERK2, and immune complex kinase assays included glutathione S-transferase (GST) Jun protein as substrate. ERK2 was activated by v-Abl and v-Src (Fig. 1*A*, lanes 2 and 4), but not by p185 Bcr-Abl (lane 3) or p210 Bcr-Abl (data not shown). In contrast, JNK was activated 3- to 4-fold by v-Abl and Bcr-Abl and 5-fold by v-Src (Fig. 1*B*), comparable to activation of JNK by Ras (11, 36). The failure of Bcr-Abl to activate ERK2, also observed by Weidemann and colleagues (37), cannot be explained by lack of expression of Bcr-Abl, which was present at higher levels than v-Abl (Fig. 1*C*). Data from four independent experiments summarized in Fig. 1*D* demonstrate that Bcr-Abl preferentially activates the JNK pathway.

Because the transient transfection experiments were performed by expressing abnormally high levels of JNK, we next asked if Bcr-Abl can activate endogenous JNK. Since the Bcr-Abl translocation is found exclusively in tumors involving hematopoietic cells, we first examined the activity of endogenous JNK in murine myeloid DAGM cells transformed by Bcr-Abl. These cells are not leukemogenic and require granulocyte-macrophage colony-stimulating factor (GM-CSF) to remain viable. After introduction of either p210 or p185 Bcr-Abl, the cells are relieved of growth factor dependence (27) and grow as disseminated leukemias in *scid* mice. The levels of JNK activity in parental DAGM cells and in two independent DAGM clones expressing p185 Bcr-Abl and p210 Bcr-Abl were compared by measuring endogenous JNK activity in immune complexes. Constitutive activation of JNK by both forms of Bcr-Abl in the presence or absence of serum and GM-CSF (Fig. 2*A*) indicated that Bcr-Abl activates endogenous JNK in a cellular context relevant for leukemogenesis. Bcr-Abl also activated endogenous JNK in Rat1 fibroblasts (Fig. 2*B*), which are a useful model for examining the role of signaling pathways in transformation by *Abl* oncogenes (26).

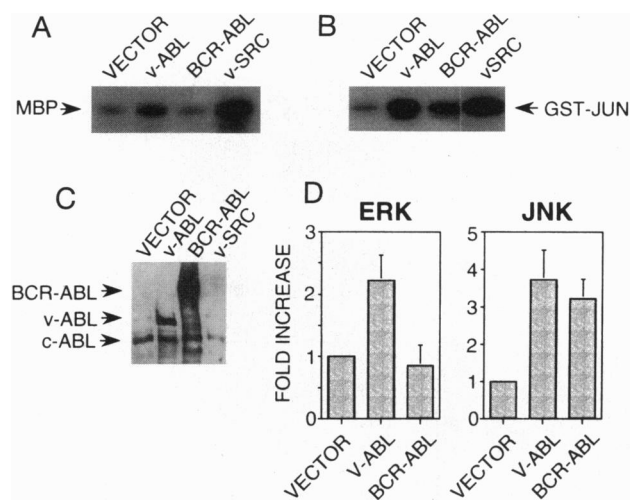


FIG. 1. Bcr-Abl preferentially activates JNK. 293 cells were transfected with 5  $\mu$ g of expression plasmids for ERK2 (*A*) or epitope-tagged JNK1 (*B*) with or without 4  $\mu$ g of expression plasmids for v-Abl, Bcr-Abl, or v-Src as indicated. Cells were incubated in the presence of serum overnight and then starved of serum 24 hr prior to harvest. (*A*) ERK activity was measured by immune complex kinase assay using MBP as substrate. (*B*) JNK activity was measured by immune complex kinase assay using GST-Jun as substrate. (*C*) Anti-Abl Western blot of whole cell lysates from cells transfected in *A*. (*D*) Summary of ERK or JNK activation by v-Abl and Bcr-Abl. Data are means  $\pm$  SD of four independent transfections and are expressed as -fold increase in activity compared to vector control. Radioactivity was quantitated with a PhosphorImager. v-Src activation of ERK (*A*) and JNK (*B*) was 5-fold above control.

**Bcr-Abl Activates Transcription from Jun-Responsive Promoters in a Ras-, MEKK-, and JNK-Dependent Fashion.** The c-Jun transcription factor can be activated by phosphorylation of its N terminus on Ser-63 and -73 by JNK (4, 5, 13). If Bcr-Abl activates JNK, Bcr-Abl should also activate c-Jun. Jun mRNA levels are a useful marker of Jun activity because Jun transcription is regulated by a TRE, the binding site for the Jun/Fos heterodimer, in its promoter (28). c-Jun mRNA levels were markedly elevated in DAGM cells expressing Bcr-Abl (Fig. 3*A*), consistent with its effects on JNK. Using a minimal

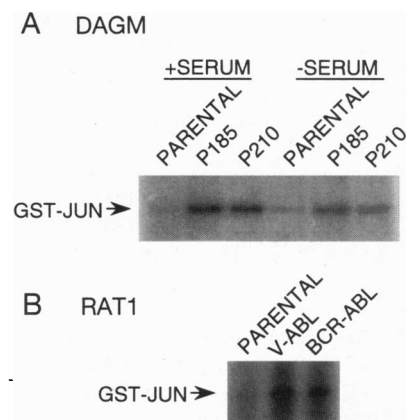
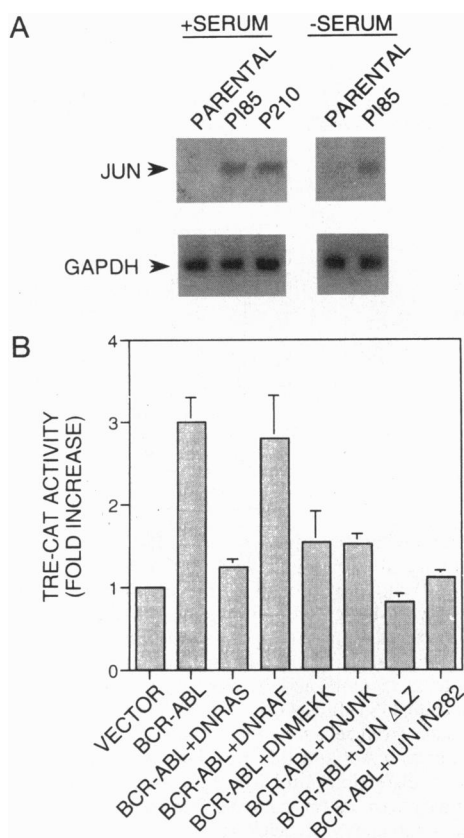


FIG. 2. Endogenous JNK activity is elevated in hematopoietic cells and fibroblasts expressing Bcr-Abl. Endogenous JNK activity was measured by immune complex kinase assay using polyclonal anti-JNK antibody (provided by Roger Davis) in  $5 \times 10^6$  parental DAGM cells or in DAGM cells expressing either the p185 or the p210 forms of Bcr-Abl (*A*) or in Rat1 fibroblasts expressing p185 Bcr-Abl or v-Abl (*B*). DAGM cells were incubated in the presence of serum (and GM-CSF for parental cells) or starved of serum (and GM-CSF for parental cells) for 3 hr prior to harvest. Rat1 cells were incubated in the presence of serum.



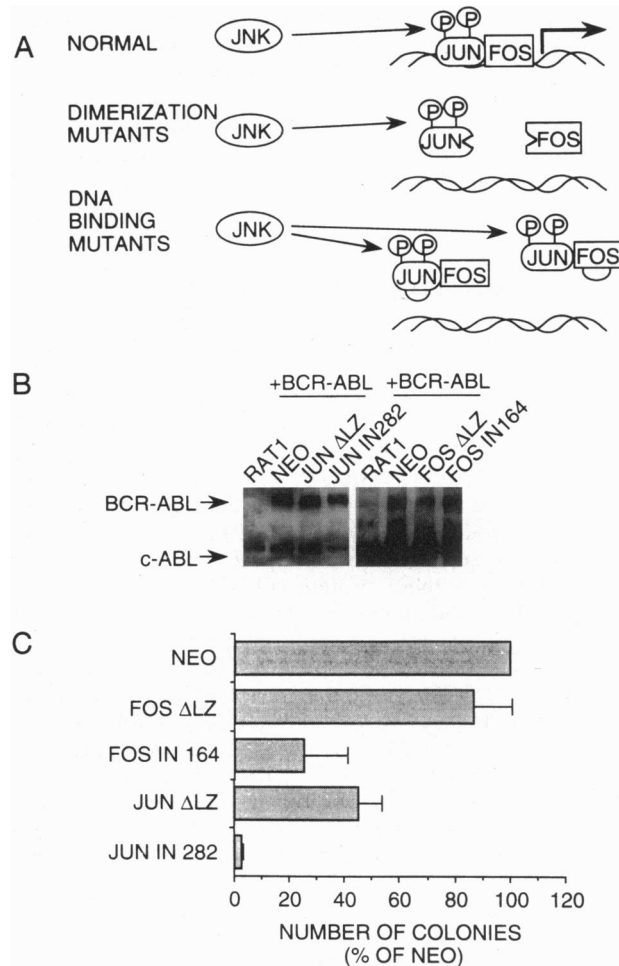
**FIG. 3.** Bcr-Abl activates Jun in a Ras-, MEKK-, and JNK-dependent fashion. (A) Cytoplasmic RNA (15  $\mu$ g) from parental DAGM cells or DAGM cells expressing either the p185 or p210 forms of Bcr-Abl was size fractionated on agarose/formaldehyde gels and probed with a murine Jun cDNA and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA as internal control. Cells were incubated in the presence of serum and (GM-CSF for parental cells) or starved of serum and GM-CSF for 3 hr prior to harvest. (B) 293 cells were transfected with 0.25  $\mu$ g of the AP-1-dependent transcriptional reporter 3 $\times$  TRE-CAT with or without 3  $\mu$ g of Bcr-Abl expression plasmid in the presence or absence of 9  $\mu$ g of dominant-negative (DN)RAS, DNRAF, DNMEKK, or DNJNK1 or 6  $\mu$ g of JUN  $\Delta$ LZ or JUN In282 expression plasmids. Cells were starved of serum beginning 20 hr after transfection and lysed after 48 hr; CAT activity was measured with [ $^{14}$ C]chloramphenicol as substrate. Percentage conversion to acetylated forms was quantitated with a PhosphorImager. Data are means  $\pm$  SE of three to six independent transfections and are expressed as -fold increase in activity compared to vector control. In the absence of Bcr-Abl, dominant-negative mutants did not significantly alter basal TRE-CAT activity.

promoter containing three TRE sites fused to the CAT gene (TRE-CAT), Bcr-Abl activated TRE-CAT 3-fold in 293 cells (Fig. 3B, lanes 1 and 2). Therefore, Bcr-Abl leads to an increase in AP-1 activity in the same cells in which Bcr-Abl activates JNK.

As reported for the epidermal growth factor and nerve growth factor RTKs (11), Jun might be activated by the MEKK/SEK/JNK pathway, using Ras as an input signal. We tested the role of Ras, MEKK, and JNK in the TRE-CAT assay by including plasmids expressing dominant-negative mutants of each protein in the cotransfection mixture. Induction of TRE-CAT activity by Bcr-Abl was efficiently blocked by dominant-negative mutants of Ras, MEKK, and JNK (Fig. 3B). In contrast, a kinase-inactive mutant of Raf, which inhibited activation of the TIS10 promoter (30) by v-Src (data not shown), did not significantly impair the Bcr-Abl effect. The results support the conclusion from a prior report that Raf activates the ERK pathway and does not activate the JNK

pathway directly (11). We therefore suspect that the Bcr-Abl signal to JNK flows through Ras, as proposed for RTKs.

**Dominant-Negative Mutants That Inhibit c-Jun Block Transformation by Bcr-Abl.** The data above raise the possibility that the transforming activity of Bcr-Abl is mediated through a Jun-dependent pathway. We therefore tested previously characterized mutants of Jun and Fos (32) for their ability to block Bcr-Abl transformation. The normal pathway of Jun activation through binding to JNK (4, 38), phosphorylation by JNK, complex formation with Fos (39), and binding to DNA is depicted in Fig. 4A. Jun In282 (Fig. 4A Bottom) is a DNA binding mutant containing an insertion in the basic



**FIG. 4.** Dominant-negative mutants of Jun and Fos inhibit transformation by Bcr-Abl. (A) Schematic of Jun activation by JNK and effects of Jun and Fos mutants. Normal pathway of Jun activation through phosphorylation by JNK, complex formation with Fos, and binding to DNA is shown (Top). Dimerization mutants Jun  $\Delta$ LZ and Fos  $\Delta$ LZ are shown (Middle). Note that Jun  $\Delta$ LZ retains the JNK binding domain and phosphorylation sites. Jun In282 and Fos In164 (Bottom) contain insertions (semicircles) in the basic regions of Jun and Fos, respectively, which disrupt DNA binding. (B) Rat1 cells overexpressing a neo control vector, Fos  $\Delta$ LZ, Fos In164, Jun  $\Delta$ LZ, and Jun In282 were infected with Bcr-Abl retrovirus and assayed for colony formation in soft agar. An anti-Abl immunoblot of representative whole cell lysates from Rat1/Neo cells or cells expressing Jun or Fos mutants 48 hr after infection with Bcr-Abl retrovirus is shown. (C) Summary of Bcr-Abl transformation of Rat1 cells overexpressing mutant Fos and Jun proteins. Transformation assays were repeated twice with two independently derived sets of indicator lines for a total of four experiments. Data are means  $\pm$  SE of four independent infections and are expressed as percentage colony formation compared to the Rat1/Neo control. Mean number of colonies ( $\pm$ SE) in the Neo control plates was  $180 \pm 43$ . No colony formation was seen for any of the indicator lines in the absence of infection with Bcr-Abl retrovirus.

region. Jun  $\Delta$ LZ (Fig. 4A Middle) is a dimerization mutant that retains the N-terminal JNK binding domain but cannot dimerize with endogenous Jun or Fos because of deletion of its leucine zipper. Similar Jun mutants that bind to JNK but cannot dimerize have been shown to act as dominant-negative proteins by competing with endogenous Jun for phosphorylation by JNK (40). As expected, both mutants inhibited the activation of TRE-CAT by Bcr-Abl (Fig. 3B). For transformation studies, analogous mutants of c-Fos were included. The Fos mutant containing a deletion of its leucine zipper (Fos  $\Delta$ LZ) served as a control because it cannot dimerize with Jun or interfere with JNK.

The effect of these mutants on Bcr-Abl transforming activity was measured in a fibroblast transformation assay in which changes in Bcr-Abl transformation can be accurately quantitated (26, 41). We derived indicator lines expressing each Jun or Fos mutant or Neo alone by retrovirus infection and selection in G418, and expression from the mutant Jun or Fos constructs above endogenous levels was confirmed (data not shown). Dominant-negative proteins had no toxic or growth inhibitory effects on the cells. No reduction in the number of G418-resistant colonies compared to the Neo control was seen during drug selection; expression of the mutant Jun and Fos constructs was uniformly stable; and there were no significant differences in cell doubling time. Therefore, any effect the dominant-negative proteins might have on *Abl* oncogene transformation can be attributed to interference with a transformation pathway rather than growth inhibition.

Each indicator line was infected with retrovirus expressing p185 Bcr-Abl or vector alone and plated in soft agar to measure the effect on transformation. Immunoblots performed on whole cell lysates showed comparable levels of Bcr-Abl expression prior to plating in soft agar (Fig. 4B), confirming that all populations were at equivalent risk for transformation by Bcr-Abl. After 2 weeks, colonies were counted and compared to the Rat1/Neo control. Four experiments with two independently derived sets of indicator lines showed that the transforming activity of Bcr-Abl was suppressed 50–90% by the dominant-negative mutants. The Fos  $\Delta$ LZ mutant, which does not affect Jun activity, had no effect. Jun In282 was the strongest inhibitor, consistent with its ability to inactivate Jun, Fos, and JNK. When Jun In282 and Bcr-Abl were simultaneously introduced into Rat1 cells using a two-gene retrovirus vector (21), we observed a similar reduction in colony number when compared to Neo and Bcr-Abl (data not shown). Thus, fibroblast transformation by Bcr-Abl is dependent on functional Jun protein.

## DISCUSSION

Studies of RTK signaling have focused primarily on the role of the Ras/Raf/MEK/ERK pathway in relaying signals from outside the cell to the nucleus (1). The human leukemia oncogene *Bcr-Abl*, although located in the cytoplasm, resembles RTKs because it dimerizes to activate its tyrosine kinase (41) and requires Ras for transforming activity (20, 21). In this report we provide biochemical and functional evidence that *Bcr-Abl* activates the JNK pathway. The results using dominant-inhibitory mutants of MEKK and JNK to block TRE activation by Bcr-Abl indicate that Bcr-Abl functions through the MEKK/SEK/JNK pathway to activate Jun. The precise details of how Bcr-Abl initiates the signal to JNK will require further study. The fact that dominant-negative Ras inhibits TRE activation by Bcr-Abl suggests that the signal flows, at least in part, through Ras. Based on recent reports that Rac1 and Cdc42 activate JNK (42, 43), it is also possible that Rho family proteins play a role.

It is curious that Bcr-Abl does not efficiently activate ERK2, whereas v-Abl does. One explanation might be the higher specific activity of the v-Abl kinase; however, we and others

(37) fail to see ERK2 activation by Bcr-Abl even when expressed at very high levels. Alternatively, structural differences between the two proteins may affect access to components of the ERK pathway. v-Abl contains a myristoylation sequence at its N terminus, which directs binding to the inner surface of plasma membranes, whereas Bcr-Abl does not. Subcellular localization experiments have shown that Bcr-Abl colocalizes with actin filaments (44), whereas v-Abl is present diffusely in the cytoplasm. Bcr-Abl and v-Abl also differ in the range of molecules they bind. Bcr-Abl binds Grb-2 (19) and a member of the 14-3-3 protein family (45), whereas v-Abl does not. Complex formation with either protein could influence activation of ERK.

Despite growing evidence linking the biochemical activation of JNK to a number of distinct stimuli ranging from RTKs (4, 11), heterotrimeric G proteins (46), tumor necrosis factor  $\alpha$ , and UV light (4, 5, 11, 13), the physiologic role of this pathway remains undefined. Our finding that inhibition of the endpoint of the pathway with c-Jun mutants impairs Bcr-Abl transforming activity supports a role for the JNK pathway in transformation. Further studies that selectively inhibit the ERK and JNK pathways in a range of cell types are required to fully define the roles of each in transformation. Such experiments are currently problematic because strategies to block ERK activity inhibit growth of normal cells and may be toxic (47), and inhibitory mutants of JNK are inefficient. Resolution of this issue is critical because the JNK pathway may be an important target for Philadelphia chromosome leukemias as well as other cancers. Selective inhibition of the JNK pathway may provide a favorable balance between therapeutic benefit and toxicity.

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